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13. ABSTRACT (Maximum 200 Words)

In the period covered by this application we have repeated a large scale animal study to investigate the effect of chronic exposure of PC-346C^{RFP} (which expresses the wild type receptor) to Casodex. The intermediate criteria we are monitoring are primary tumor volume, rates of proliferation (as measured by BrdU incorporation) and apoptosis as measured by TUNEL staining. We have standardized an efficient methodologies for isolating cells from primary tumors expressing RFP by fluorescence activated cell sorting (FACS) and by laser capture micro-dissection (LCM). We have intitiated a comprehensive micro-array based bioinformatics effort to identify genes whose expression is modulated by Casodex to characterize the molecular events underlying metastatic progression of PC-346C^{RFP} cell. The changes in gene expression detected by micro-array are being validated by QT-PCR using SYBR green. The results of these experiments are also being compared to the effects of Casodex in LNCaP cells (which has a mutated androgen receptor), and to the changes in gene expression seen in the PC-346^{RFP} primary tumors (and metastases) from animals chronically treated with Casodex. These studies will identify changes in cell behavior and gene expression that lead to the acquisition of an invasive phenotype.

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Prostate cancer, casodex, metastasis, apoptosis, gene array

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Introduction

In 2000, an estimated 180,4000 men were diagnosed with prostate cancer in the United States, and 31,400 succumbed to the disease (American Cancer Society Facts and Figures, 2000). Due to the increase in public awareness and the greatly increased use of Prostate Specific Antigen (PSA) for screening, prostate cancer now is the second most commonly diagnosed male cancer in many western countries after lung cancer. The major risk factors for prostate cancer include age and race, and the consumption of a high fat diet. The main cause of death from prostate cancer is the invasion and metastasis of prostate cancer to the bone, liver and brain. However, for many men (approximately 100,000 of those diagnosed each year) the disease will remain localized and slow growing. Extensive PSA screening programs have lead to the increased identification of early stage (A1 and A2) tumors in younger men. Approximately 70% of these tumors are indolent and will not need treatment during the patients life time (Choo et al., 2002). Unfortunately at present there is no way to distinguish between aggressive, clinically significant tumors that need to be treated and indolent tumors. As a result, many patients are treated more aggressively than is necessary.

There are four major strategies for treatment of localized, early stage prostate cancer: radical prostatectomy, radiation therapy (either external beam, three dimensional conformal therapy or brachytherapy), hormone therapy (usually with Casodex or flutamide with or without an LH-RH agonist such as Zoladex) and watchful waiting (waiting for the PSA levels to rise before deciding on a course of treatment). The combined five year survival for these interventions is approximately 75 %, however the majority of recurrent tumors develop resistance to further therapeutic intervention. The recent Bicalutamide 150mg (Casodex) Early Prostate Cancer (EPC) Program was established to examine whether adding 150mg/day Casodex immediately to standard care (watchful waiting, radical prostatectomy or radiotherapy) reduces the risk of disease progression and improves survival when compared to standard care alone. Analysis of the data from the EPC Program, which enrolled 8,113 patients with localized and locally advanced prostate, shows that Casodex cuts the risk of disease progression by almost half in patients with localized or locally advanced prostate cancer, and also demonstrates that the time to prostate-specific antigen (PSA) doubling was significantly delayed in patients receiving Casodex and standard care compared with standard treatment alone (Wirth et al., 2001; Wirth, 2001; Iversen et al., 2002). As a result, there is a very significant increase in the number of patients being treated with Casodex, either alone or immediately after surgery or radiation. Furthermore, neoadjuvant therapy with Casodex to debulk organ-confined prostate tumors (particularly stage B1) and to improve positive margins is now widely used prior to surgery and radiation therapy (Padula et al., 2002), and many 'at risk' men (defined as men with two first degree relatives with prostate cancer) are now considering chemoprevention in the form of Casodex (Trump et al., 2001; Schellhammer, 2002).

The aim of the studies funded by this award is to examine the effects of Casodex and other anti-androgens on the induction of apoptosis in androgen dependent PC-346C and LNCaP human prostate cancer cells, and to understand the molecular basis of tumor progression. These cell lines are being used as a model of early, organ confined androgen dependent prostate cancer. One of the major unresolved issues in the development of prostate cancer is the mechanism underlying the progression from hormone dependent to hormone refractory prostate cancer after treatment with anti-androgens. Since there are an increasing number of men being treated with Casodex mono-therapy for localized prostate cancer, as a result of the initial success of the 150mg (Casodex) Early Prostate Cancer (EPC) Program, it is important to fully evaluate the biological effects of Casodex to ensure that it does not induce adverse effects.

Body

The experimental aims for this operating grant are:

Task 1: Analysis of PC-346C cells (months 1-8) to determine the effects of Casodex on apoptosis and cell cycle, determine whether Casodex or flutamide can induce an invasive phenotype, to monitor changes in gene expression using RT-PCR and to clonally expand the invasive cells for further study.

Task 2: Determine the metastatic capability of the invasive cell lines produced above, both in vitro and in vivo using the orthotopic xenograft model system. (months 8-20).

Task 3: Examine the induction of the invasive phenotype in LNCaP^{GFP} and PC-346^{RFP} cells, and to characterize the changes in gene expression induced by Casodex. (months 8-20). Initiated, in progress

Task 4: Identify differentially expressed genes using microarray technology (months 3 -36)

As reported in the last annual report (2004) the experiments outlined in **Task 1**, have been completed on schedule, and we have published two manuscripts (Zhan et al., 2003 and Lee et al., 2003).

With regard to the experiments outlined in **Task 2**, we previously reported that the LNCaP sublines, I-1 and I-33, when grown as xenografts in nude mice grow slowly as well encapsulated primary tumors that metastasized infrequently to other organs (4/50 animals for each subline). We first isolated these cell lines from the invasive LNCaP population that transversed the 8μ membranes in the Boyden chamber assay. *In vitro* these cell lines grow quite rapidly and are consistently very invasive, however in the xenograft model these cells form relatively slow growing tumors and do not appear to be particularly aggressive. There are several possible reasons for this low rate of metastasis: First, in contrast to their invasive phenotype *in vitro*, these cells may not be intrinsically metastatic *in vivo*. This would suggest that the acquisition of an invasive phenotype is reversible and is dependent on either intrinsic or extrinsic factors to maintain the invasive phenotype. Secondly, it is well established that the mutation of the androgen receptor present in the LNCaP cells renders the receptor promiscuous, and results in the agonistic activation of the receptor by dehydroepiandrosterone (DHEA). This activation by DHEA may block or severely blunt the signaling by Casodex that leads to the induction of apoptosis and metastasis. Thus as we reported last year, the use of LNCaP cells may confound the successful completion of Task 2 as it was originally conceived. As stated last year we have modified the original SOW to see if we can resolve this issue by generating a new set of cell lines which express the wild type androgen receptor tagged with red fluorescent protein (PC-346^{RFP}). As we reported last year this cell line undergo cell cycle arrest and apoptosis in a time and dose dependent manner in response to Casodex that is essentially indistinguishable from the parental cell line. These cells have been used to establish an orthotopic xenograft model of localized prostate cancer expressing the wild type androgen receptor that responds to Casodex treatment in doses that are equivalent to those produced the 150 mg Casodex. We have demonstrated that implantation of Casodex (50mg sustained release 90 day pellets) into androgen replete nude mice induces significant tumor regression, through cell cycle arrest and apoptosis, and induces significant changes in angiogenesis in the primary tumor. When grown as orthotopic tumors however, these cells do not appear to metastasize after treatment with Casodex. Using Laser Capture Micro-dissection (LCM) and Fluorescence Activated Cell Sorting (FACS) to purify the PC-346C^{RFP} cells from tumors. Using reverse transcriptase polymerase chain reaction (RT-PCR), western analysis and immunofluorescence we have shown that the expression of Red Fluorescent Protein (RFP) is unaffected by treatment with Casodex (data not shown). Furthermore, even though the tumors undergo significant regression, the expression of the AR in the remaining tumor is essentially

unaffected, either in its level or nuclear localization. This is in marked contrast to the *in vitro* data that has shown that expression of the AR is decreased in both LNCaP and PC-346C cells after treatment with Casodex and the receptor is relocalized to the cytoplasm (Lee et al., 2003). As we reported last year when grown in the presence of growth factor reduced Matrigel and treated with Casodex (50mg sustained released 90 day pellets), the orthotopic tumors showed signs of metastatic progression to the lymph nodes, epididymis and subcutaneous sites, as evidenced by the presence of RFP staining in the metastatic deposits. This pilot study utilized 5 animals for each of the experimental groups, and even though 3/5 animals developed metastases in one or more sites was not large enough to reach statistical significance.

During the last reporting period we have repeated this experiment with a larger number animals, using a significantly smaller inoculum of cells (50,000 cells versus $2x10^6$ cells in the initial experiments). These two modifications have helped to resolve the major shortcomings of the previous experiments. First we have reduced the influence of the growth factors present in the Matrigel; secondly we have removed the interference by adrenal steroids; third we have extended the time that the primary tumors can be exposed to Casodex before the tumor size induces unacceptable morbidity from about 5 weeks to more than 20 weeks. This experiment is being repeated with the same experimental design using 15 animals per group, at several time points.

While the experiment has yet to be completed, it is clear from the gross morphology of the tumors and the appearance of metastases, that extended exposure to Casodex does induce a metastatic phenotype in PC-346C^{RFP} cells (see table 1).

	Weeks			
Cell Line	3	9	12	15
PC-346C^{RFP}	0/15	0/15	0/15	06/06/05
PC-346C^{RFP} +Casodex	0/15	1/15	3/15	06/06/05
PC-3	0/15	5/15	06/01/05	06/22/05

Table 1: Analysis of metastasis by gross morphology of liver and/or lungs for metastases. 50,000 PC-346C^{RFP} or PC-3 cells were orthotopically inoculated into the prostate of male nude mice supplemented with testosterone. PC-3 cells, which are known to metastasize to the liver and lungs were used as positive controls. [Dates on which remaining animals are to be sacrificed is shown].

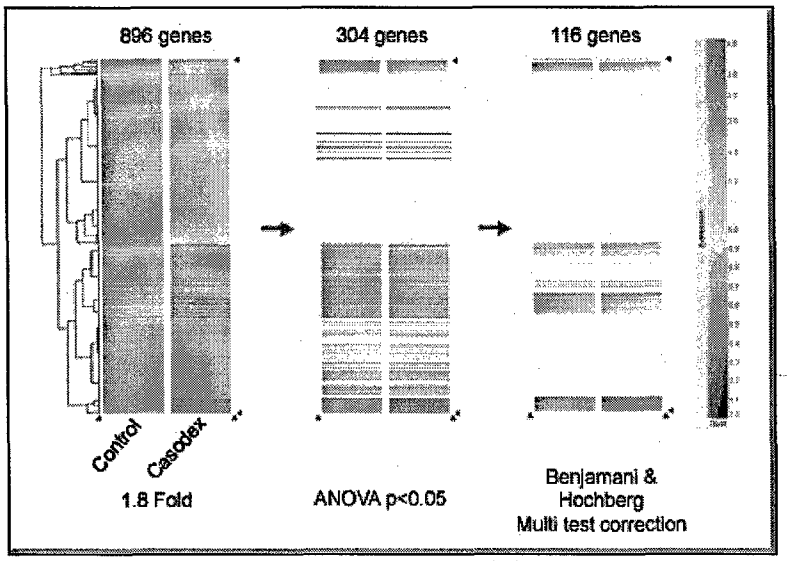

Figure 1: Gene array analysis of PC-346C^{RFP} treated with Casodex. Each lane represents the average of 3 arrays for each treatment group, providing a 3x3 matrix for analysis.

Task 4: We have compared the steady state mRNA levels in PC-346C cells before and after treatment with Casodex using the Nimbelgen human genome array containing 38,000 genes.

The microarray was performed using 3 independent RNA samples from control and treated PC-346C cells, providing a 3X3 array matrix.. Using the proprietary GeneSpring Software we have identified 896 genes that are up- or down-regulated by greater than 1.8 fold. Subsequent stringent statistical analysis using ANOVA and the Benjamani and Hochberg mutliple test correction, reduces the number of genes whose expression is significantly regulated in PC-346C cells after treatment with Casodex to 116 genes. Inspection of these genes shows that 28 are uncharacterized Open Reading Frames (ORF) with no defined function, and 23 sequences that represent hypothetical ORFs, leaving an array of 65 genes of known function whose expression is significantly altered by treatment with Casodex. As shown in Table 2, these genes can be binned into several groups related to fundamental biological processes.

Process	#	upregulated	downregulated
Apoptosis	4	p8, tribbles 3	survivin, plk
Metastasis		NONE	prothymosin α, KAI 1,
Cell Cycle	14	p21(variant 2), ddit3	e2f, cdc2,cdc 20, cyclin D1, cyclin D3, cyclin E2,
DNA Replication	4	NONE	pcna, mcm7, rfc2, pola2
RNA Transcription	2	NONE	pol2, dnmt1
RNA processing	2	NONE	hnrpm 1, hnrpm 2, lsm2
Transcription factors	3	CEBP, ATF3	GTF3C5
Chromatin Structure	2	NONE	H2A, hist 1
Stress Response	1	ses2	None
Signal Transduction	2	NONE	calr , ack1
Metabolism	4	cyp1A1	smr, tk1, pck 2

Table 2: Binning of genes that are up- or down regulated in PC-346C cells in response to Casodex. Changes in gene expression have been confirmed by QT-PCR for underlined genes .

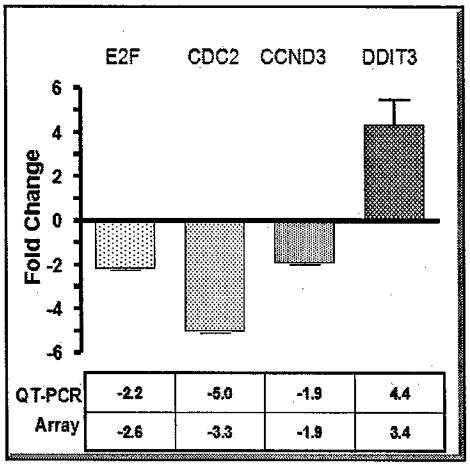

Figure 2: Changes in relative levels of transcripts associated with cell cycle progression in PC-346C cells after treatment with Casodex

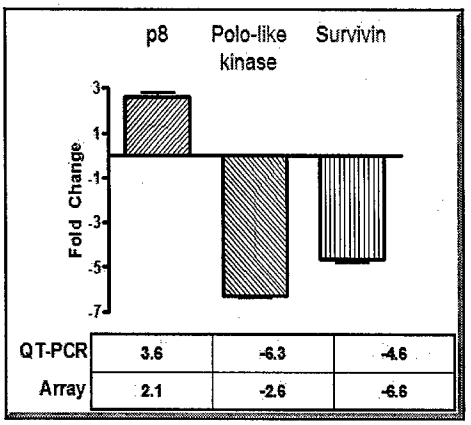

Figure 3: Changes in relative levels of transcripts associated with apoptosis in PC-346C cells after treatment with Casodex

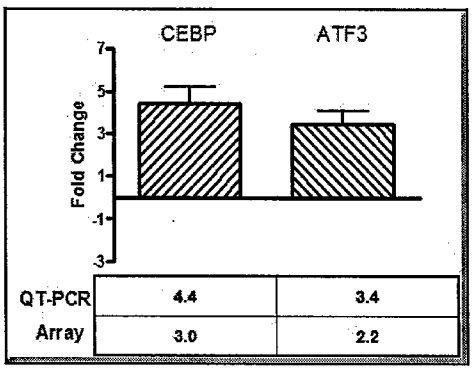

Figure 4: Changes in relative levels of transcripts associated with transcription in PC-346C cells after treatment with Casodex

As exemplified in Figures 2-5, we are now confirming the changes in gene expression using SYBR-green QT-PCR for genes binned into each functional category. Using RNA isolated from PC-346C^{RFP} cells 48h after treatment with vehicle or 50μM Casodex (the same time and dose used for the gene arrays). As shown in the lower panel of each figure there is generally excellent concordance between the gene array and the QT-PCR data.

We have established the protocols needed for the preparation of RNA for gene array from samples prepared from frozen orthotopic tumors before and after treatment with Casodex. To facilitate these experiments and eliminate variability due to tumor composition which would confound the data analysis, we have developed a very efficient methodology for isolating the human prostate cancer cells from the primary tumor (where they may be contaminated with host stroma), and from metastatic sites (where they may be contaminated with both host stroma and epithelium). This methodology is based on Fluorescence Activated Cell Sorting (FACS) on a Beckman-Coulter ALTRA FACS, as outlined in the previous annual report.

To provide a more complete picture of the effects of Casodex on gene expression in the both the primary orthotopic tumors and metastatic deposits, RNA samples from each of the tumors are now being prepared and will be interrogated by QT-PCR to determine the changes in a battery of approximately 20 genes selected from the validated genes determined as described above, standardized against GADPH and actin.

Key Research Accomplishments

- Characterization of cellular pathways involved in induction of apoptosis after anti-androgen therapy in PC-3465C^{RFP} cells in vitro, with particular emphasis on the role of the mitochondria (documented in Lee et al., 2003 and Zhan et al., 2002)

- Publication of two review papers germane to this project, both of which reference the central hypothesis being tested in the experiments outlined in this report (Lee and Tenniswood 2004a, 2004b).

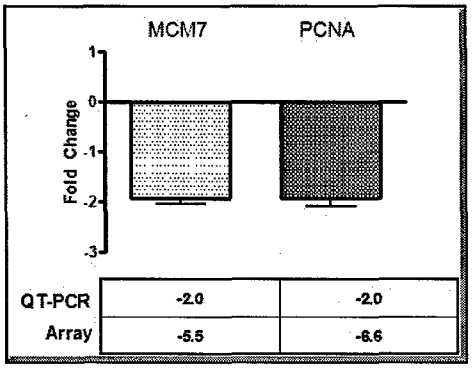

Figure 5: Changes in relative levels of transcripts associated with replication in PC-346C after treatment with Casodex.

- Demonstration that invasive sublines of LNCaP cells are not highly metastatic in vivo, (probably due to the high levels of adrenal steroid, DHEA, in the rodent host).

- Establishment and refinement of new model of androgen dependent anti-androgen responsive localized prostate cancer.

- Demonstration that treatment of orthotopic tumors derived from PC-346C^{RFP} cells induce apoptosis in response to Casodex

- Demonstration that Casodex treatment can also induce metastatic progression in the PC-346C^{RFP} cells, providing support for the suggestion that the LNCaP cell line and its derivatives may not be ideal model cell lines for orthotopic studies, and providing a possible explanation for the failure of the invasive LNCaP cells to metastasize.

- Development of a robust methodology for the isolation of RFP tagged cells from orthotopic tumors (primary and metastatic) that can be used for Gene array analysis and Western analysis.

- Documentation of the changes in gene expression (<1.8 fold) induced by casodex, and verification of the changes by QT-PCR.We have identified 65 genes that are differentially regulated by Casodex in PC-346C cellsand have confirmed the changes in gene expression of 20 of these genes todat by QT-PCR. The remaining genese will be analyzed in the near future.

Reportable Outcomes

- We have created a number of novel cell lines including:

PC346C^{RFP}
LNCaP^{RFP}
PC346C^{GFP}
DU-145^{RFP}
DU-145^{GFP}

- We have designed, synthesized and validated pimers for QT-PCR for 20 independent genes. These sequences are being archived t the present but will be submitted to the NCBI database once they have been validated.

Since the last annual report 3 manuscripts have been published which acknowledge the support of DAMD17-01-1-0114:

Lee, E.C.Y. and Tenniswood, M. (2004) Emergence of Metastatic Hormone Refractory Disease in Prostate Cancer after Anti-androgen Therapy. *Journal of Cellular Biochemistry* 91:662-670.

Chrenek, M., Erickson T., Gee, C. Lee E.C.Y., Gilmore K.,Tenniswood, M. and Wong, P. (2004) Comparative Functional Genomics: Analysis of Changes in mRNA Profiles in Multiple Model Systems for Understanding Basic Biological Phenomenon. *Transactions of Integrated Biomedical Informatics and Enabling Technologies* 1:43-54. (epub).

Tenniswood, M. and Lee, E.C.Y. (2004) On the Trail of Cell Death Pathways in Prostate Cancer. *Cancer Biology and Therapeutics*. 3:779-771.

In addition the following manuscript has been submitted for publication:

Lee, E.C.Y., Ayala, G., Flanagan, L., Packman K., Van Weerden, W., Romijn, J. and Tenniswood, M. (2005) Characterization of Casodex-Responsive Orthotopic Xenografts of Androgen Receptor Positive PC-346C^{RFP} Human Prostate Carcinoma Cells. (Submitted to Urology)

Conclusions

The experiments completed to date provide *in vitro* and *in vivo* support for the hypothesis that treatment of localized prostate cancer with Casodex or use of the drug in a chemo-preventive setting, may be inappropriate and lead to a higher tumor burden of androgen independent metastases following treatment. Completion of the pre-clinical examination of the effects of Casodex on the molecular and cell biology of PC-346C cells in the absence and presence of Casodex may provide the explanation for the recently observed increase in the number of deaths in the which have lead to the recommendation by Health Canada to discontinue Casodex monotherapy for prostate due to the trend toward accelerated deaths compared to placebo.

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Trump DL, Waldstreicher JA, Kolvenbag G, Wissel PS and Neubauer BL (2001) Androgen antagonists: Potential role in prostate cancer prevention. *Urol* 57: 64-67.

Schellhammer, PF (2002) An evaluation of bicalutamide in the treatment of prostate cancer. Expert Opin Pharmacother 3:1313-1328.

Lee, E.C.Y. and Tenniswood, M. (2004) Programmed Cell Death and Survival Pathways in Prostate Cancer Cells. *Archives of Andrology* 50:27-32.

Zhan P, Lee, ECY, Packman, K and Tenniswood, M (2002) Induction of Invasive Phenotype by Casodex in hormone sensitive Prostate Cancer Cells. *J Steroid Biochem Mol Biol* 83: 101-111.

Lee, ECY, Zhan, P, Packman, K and Tenniswood, M (2003) Anti-androgen induced cell death in LNCaP Human Prostate Cancer Cells. *Cell Death and Differentiation* 10:761-771

Montpetit, ML and Tenniswood M (1989) Separation of mature rat ventral prostate epithelial and stromal cells. *Prostate* 15: 315-325.

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PROGRAMMED CELL DEATH AND SURVIVAL PATHWAYS IN PROSTATE CANCER CELLS

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Programmed cell death, or apoptosis, is a series of morphologically and biochemically related processes. The extrinsic (death receptor mediated) and intrinsic (mitochondrial-mediated) apoptotic pathways can be triggered by physiological and pharmacological substances. However, other molecular events influence the sensitivity of prostate cancer cells to apoptotic stimuli, leading to marked variations in the responsiveness of prostate cancer cell lines to individual stimuli. Modulation of apoptotic responses by over expression of anti-apoptotic proteins (NF-κB, IAPs and Bcl-2), or attenuation of pro-apoptotic proteins (PTEN and Bax) may be responsible for the variations in sensitivity of these cell lines to hormone and chemotherapy. The expression of anti- and pro-apoptotic proteins in some of the widely used in vitro models of prostate cancer is reviewed.

Keywords apoptosis, refractory cancer, PTEN, IAP

INTRODUCTION

Most therapeutics for prostate cancer activate the apoptotic machinery in prostate cancer cells. Regardless of the initiating event, epithelial cells from the normal prostate and most tumor cells undergo similar morphological processes, although the timing of the events may vary from cell to cell (Fig. 1). Apoptosis in the prostate is initiated by either extrinsic pathways or intrinsic pathways or a combination of these pathways (Fig. 2).

In addition to the appearance of apoptotic bodies, there are several key apoptotic events that can be used to monitor programmed cell death. This includes DNA Fragmentation, Annexin-V staining and caspase activation. Assessment of apoptosis using these endpoints, however, need to be used with some caution.

1. *DNA Fragmentation:* Most prostate cancer cells have the enzymatic apparatus necessary to complete DNA fragmentation [14]. However, subtle differences in intranuclear pH, or activating divalent cation concentration, affect the extent of DNA fragmentation [2]. DU145 and LNCaP cells do not completely fragment their DNA, or generate

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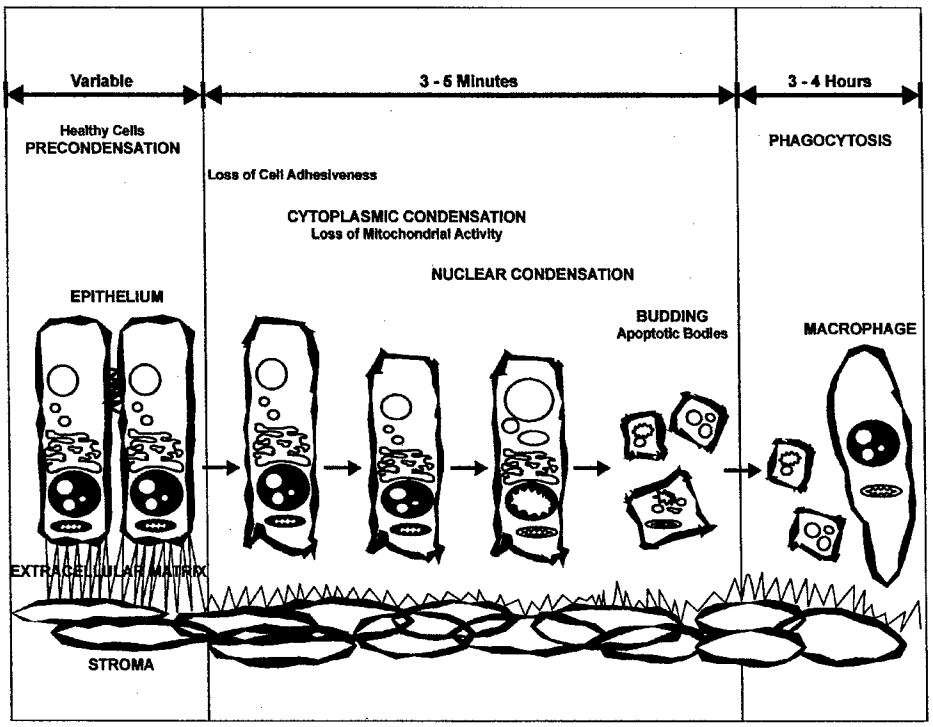

Figure 1. Schematic representation of stages of apoptosis in individual glandular epithelial cells. The process of apoptosis after hormone ablation in the prostate can be broken down into several stages. The length of the pre-condensation stage varies from cell to cell and probably reflects the micro-heterogeneity of hormone or growth factor in the environment. Cytoplasmic condensation involves the loss of the interactions between the dying cell and its epithelial neighbors, degradation of the extracellular matrix and the loss of mitochondrial membrane potential. During nuclear condensation, endonuclease activation results in the fragmentation of the DNA and its marginalization to the nuclear periphery. The fragmentation phase is characterized by the formation of apoptotic bodies, which are phagocytosed by the neighboring epithelial cells or resident macrophages.

oligonucleosome ladders after treatment with anti-androgens, even though the necessary enzymes are present in the nucleus [29].

2. *Annexin-V Staining:* In apoptotic cells the relocation of phosphatidylserine, from the inner to the outer leaflet of the cell membrane can be detected by Annexin-V. Programmed cell death in most prostate cancer cell lines can be assessed by measuring the number of cells stained positive for fluorescein-conjugated Annexin-V using fluorescence microscopy or flow cytometry [17, 22].
3. *Caspase Activation:* Activation of caspase-3, 6, 7, 8 and 9 plays a central role in apoptosis and can be used as a surrogate marker for apoptosis (Fig. 2). The activation of individual caspases is cell-type specific and also dependent on the type of apoptosis-inducing agent (11, 27).

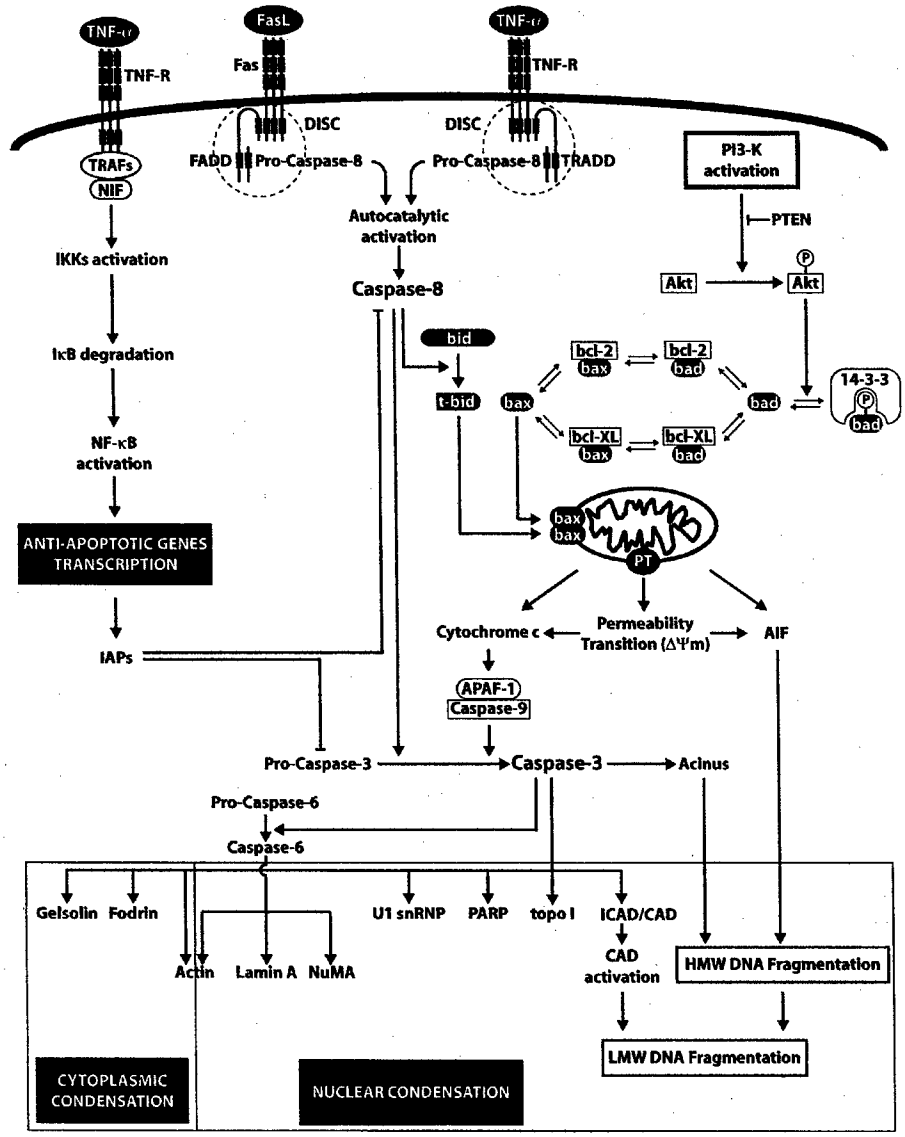

Figure 2. Extrinsic and intrinsic pathways of cell death and survival. The DISC (death-inducing signaling complex) is assembled after ligand binding to the death receptor. Autocatalytic activation of caspase-8 leads to activation of the executioner caspases and proteolysis of many substrate proteins. Activated caspase-8 also triggers the mitochondria-mediated pathway through the facilitated assembly of the mitochondrial permeability transition (PT) pore complex. This results in the dissipation of mitochondrial membrane potential ($\Delta\Psi m$), and release of cytochrome *c* and AIF from the mitochondria. The mitochondrial pathways leading to permeability transition are negatively regulated by Bcl-2 and Bcl-xL. Cytochrome c forms the apoptosome with Apaf-1 (apoptotic protease-activating factor 1) and pro-caspase 9, activating caspases 3 and 6, triggering high molecular weight (HMW), DNA fragmentation (through AIF and acinus) and oligonucleosome formation through CAD (caspase activated DNase).

Survival Pathways in Prostate Cancer Cells

Prostate cancer cell lines block apoptosis using a variety of survival mechanisms including PTEN, NFκB, IAPs and Bcl-2 family proteins, which makes it difficult to generalize the effects of cytotoxic drugs based on a single cell line. For example the ALVA-31, PC3 and DU145 cell lines are highly sensitive to apoptosis induced by TRAIL (TNF-related apoptosis-inducing ligand) through the death receptor-mediated pathways, while LNCaP cells are resistant to TRAIL (19, 25).

1. PTEN

PTEN (phosphatase and tensin homologue deleted on chromosome 10, MMAC1/TEP1) is a tumor suppressor gene that functions as a dual-specificity phosphatase in vitro (12). Although PTEN gene is transcribed in both DU145 and LNCaP cells, the protein is only active in DU145 cells due to a frame-shift mutation in the PTEN gene in LNCaP cells that is also common to malignant prostate cancer (3, 25). PTEN dephosphorylates the lipid signal transduction molecule phosphoinositide 3,4,5-trisphosphate [PI(3,4,5)P_3] and serve as a negative regulator of the signaling events mediated by phosphatidylinositol 3-kinase (PI3-K) (13). Cell lines, such as the LNCaP, lacking PTEN phosphatase activity display constitutively elevated steady-state levels of PI(3,4,5)P_3 which activates PDK1 and PDK2. This results in constitutive activation of Akt (kinase B), a survival promoting serine/threonine kinase. Intracellular signaling through this pathway suppresses apoptosis by phosphorylating and inactivating caspase-9 and pro-apoptotic Bcl-2 family member Bad (6) (Fig. 2). The loss of PTEN expression in LNCaP cells elevates Akt activity and protection from TRAIL-induced cell death by sequestering Bad from the mitochondrial membrane and blocking the activation of the executioner caspases (20). However, this regulation of the PI3-K/Akt pathway is both death receptor and cell-type specific, as the lack of PTEN expression does not render LNCaP cells insensitive to TNF or PC3 cells insensitive to TRAIL (9).

2. NF-κB and IAPs

Execution of the apoptotic pathways is also regulated by the transcription factor, NF-κB, which is normally found as an inactive cytoplasmic heterodimer complexed with its cognate inhibitor, IκB (1). Multimerization and recruitment of TRAFs (TNF receptor-associated factors) to the cell surface receptor along with NF-κB-inducing kinase (NIK) activates the IκB kinases (IKKα and IKKβ). This leads to the phosphorylation, polyubiquitination and degradation of IκB (16, 28), and the release of NF-κB which translocates to the nucleus to activate transcription of the genes responsible for the anti-apoptotic response (4). The IAP proteins suppress apoptosis by binding to and inhibiting the proteolytic activity of caspase-3, -7, and -9. The IAPs also bind to TRAF hetero-complexes, interfering with the upstream activation of pro-caspase-8 (21, 26). The activation of NF-κB and the induction of IAPs is an integral part of the negative-feed-back-control of the apoptotic machinery that protects cells from undergoing apoptosis. NF-κB is constitutively active in several androgen-insensitive prostate adenocarcinoma cell lines including DU145, PC-3, Du-Pro and TSU-Pr1, but is not in androgen-responsive cell lines such as LNCaP and CWR22RV1 (23). In cells that display constitutive activation

of the IKK complex and enhanced expression of IAPs (DU145 and PC-3 cells), lowered sensitivity towards etoposide is displayed, compared to LNCaP cells, which are very sensitive to the drug and activate NF-κB normally (15, 23). This suggests that the differences in NF-κB and IAP activation pathways may be responsible for the differential sensitivity of prostate cell lines to chemotherapeutic agents.

3. Bcl-2 Family

Anti-apoptotic members of the Bcl-2, such as Bcl-2 and Bcl-xL, block the cell death process through their protective role in mitochondrial integrity (Fig. 2) (10). These proteins heterodimerize and neutralize the pro-apoptotic activity of other Bcl-2 family proteins such as Bax and Bad. They also protect the mitochondria against the loss of permeability transition and block the release of cytochrome *c* and AIF (apoptosis inducing factor) from the intermembrane space to the cytosol. Bcl-2 overexpression is correlated with the emergence of hormone refractory disease and the progression of metastatic disease in prostate cancer (5). However, there is no correlation between endogenous Bcl-2 levels and androgen-responsiveness in different prostate cancer cell lines (8, 18, 24), nor is there a correlation between endogenous Bax expression levels and the status of androgen-sensitivity since only DU145 has been shown to be Bax-deficient (7). Thus, abrogation of the Bcl-2-regulated mitochondrial-mediated pathway is not entirely responsible for the emergence of androgen-resistance phenotype after hormone therapy.

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COMPARATIVE FUNCTIONAL GENOMICS: ANALYSIS OF CHANGES IN mRNA PROFILES IN MULTIPLE MODEL SYSTEMS FOR UNDERSTANDING BASIC BIOLOGICAL PHENOMENON*

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Abbreviations: TAM – Tamoxifen; ICI – ICI 182,780; CAS – Casodex (Bicalutamide); SERM – selective estrogen receptor modulator

We are interested in the molecular conditions within cells that sensitize them to active cell death (apoptosis). This is important in the study of cancer and other biological conditions because by understanding what makes cells die normally, we will better understand how to approach disease states cause by the disruption of these processes. We used a gene macroarray approach to analyze changes in gene expression in breast cancer cells in culture when they were treated with Tamoxifen (TAM). However, rather than using the pairwise, n-fold comparison of mRNA abundances that has become standard with array analyses, we used what we term a comparative functional genomics approach. With this approach, we were looking for consistent changes in gene expression that were conserved in multiple models of inducible apoptosis. In the current study, we examined breast cancer cells treated with TAM, ICI 182,780 and TNFα and prostate cancer cells treated with Casodex. Rather than arbitrarily defining an n-fold value as being significant, we were relying on concordant changes in the gene expression profiles in the different models to define which changes have a potential biological significance. In our pilot study, we designed a small array experiment and have identified four genes that may be relevant for active cell death sensitivity associated with TAM blockage of estrogen receptors.

Introduction

The current approach to the analysis of genomics and proteomics data is complex. Gene array analyses and 2D protein gel experiments produce massive amounts of data that must be interpreted to identify those pieces of data that are significant. One ends up with a very large picture of what is happening, but it is often confusing as to which part of the picture is the most important. When zoologists were attempting to unravel vertebrate anatomy, an innovative comparative vertebrate anatomy approach was developed to study isolated structural systems. We

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believe that a similar comparative approach can be applied to array analysis to reveal biologically significant changes in gene expression with respect to isolated biological events.

We are interested in a single biological phenomenon, the process of apoptosis. We have been using a differential screening approach to identify genes that are differentially expressed in an apoptotic-induced state as compared to a non-apoptotic state. This approach is useful because the profile of gene expression within a cell determines the behavior of that cell and differences in gene expression allow differences in cell behavior. With respect to traditional differential screening of a cDNA library or array, it has become standard to set an arbitrary n-fold differential expression value as the criteria for deciding what might be a biologically significant difference between a pair of conditions (for example cells treated with a specific drug versus untreated cells). This approach does not consider that there may be changes in gene expression that are not involved in the process being studied. In addition, setting high arbitrary criteria for the amount of change that is significant for gene expression does not allow one to consider that, in some cases, small changes in the expression of specific genes may be sufficient to have a biological effect.

We have developed a comparative functional genomics approach to identify changes in gene expression that are conserved between different model systems undergoing a common biological process such as apoptosis. By doing this, we are attempting to tease out the common events that define the apoptotic process by comparing consistent changes (sometimes subtle ones) that occur in these model systems. This is a different approach than traditional methods of analyzing functional genomic data. The comparative functional approach recognizes that if a biological process is used by all cells, then we can identify those genes that are important for that process by identifying which genes are regulated in a similar fashion by induction of the process in different systems. In the current manuscript, we describe our pilot screen of a small array of genes to identify estrogen receptor-mediated expression changes that are conserved in four cell culture model systems of apoptosis sensitivity.

Defining Apoptosis

Apoptosis is an active cell death process that cells activate in response to certain endogenous or environmental stimuli.[1,2] This is a naturally occurring process and is the mechanism by which unwanted tissue is removed during development, and by which damaged cells are removed.[3-5] The apoptotic processes within a cell are controlled by biomolecular balances and switches. Because different cell types respond uniquely to environmental stimuli and growing conditions, the cues to activate apoptosis may vary from cell type to cell type. However, because apoptosis is essentially an ubiquitous process, the overall repertoire of receptors, signal transducers, activators and executors within all cells must be similar. The differences in the expression of these molecules are indicative of the tailor-made balance for each cell type. Thus, apoptosis is an interesting biological process to study from a basic biological perspective as a system that can be controlled through a complex series of signaling events and a variety of inputs. It is also medically important from a number of perspectives including understanding and treatment of diseases where apoptosis is misregulated such as cancer.

Cancer and the apoptotic process

Normally, when a cell becomes dysfunctional, apoptosis is induced and the cell dies for the overall benefit of the organism.[4] Cancer cells arise from normal tissue through a series of modifications to biological cellular processes. These events include induction of cell cycle and misregulation of apoptosis as early events through to enhancement of interactions with and modifications to the extracellular environment in metastatic disease.[6]

One goal of cancer biology research is to find a way to specifically kill cancer cells. Some chemotherapeutic drugs that have been designed to treat cancer do so by sensitizing these cells to apoptosis inducing signals. Of particular interest here are the drugs Tamoxifen (TAM) and ICI 182,780 (ICI), selective estrogen receptor modulators (SERMs) that block estrogen receptors and the anti-androgen Casodex (CAS) that blocks androgen receptors.[7-9]

Tumors and cell culture systems

The majority of breast tumors are derived from mammary gland epithelial cells. These tumors start as ductal carcinoma *in situ* and progress through invasive carcinomas to metastatic disease.[10] Mammary epithelial cell survival is normally dependent upon the presence of estrogens. Many kinds of breast cancers derived from these cells are also dependent upon estrogen for growth and to enhance resistance to apoptosis. Molecules that bind intracellular estrogen receptors and prevent proper functioning of the receptor can either induce apoptosis or produce

a cell that is more sensitive to apoptosis inducing signals.[7] In the current study, we recapitulate these effects in culture by treating MCF7 cells, an estrogen dependent human mammary epithelial cancer cell line, with the SERMs TAM and ICI.

Prostate tumors are generally derived from androgen dependent prostate epithelial cells.[11,12] Some prostate cancers and cancer derived cell lines are also dependent upon androgen for normal function. Apoptosis can be induced in these cells if the androgen receptor is blocked by an anti-androgen that prevents proper functioning of the receptor. We mimic these effects in culture by treating PC346C cells, an androgen dependent human prostate tumor derived cell line, with CAS.

Cell culture systems are used in our analysis because the response of single cell types is difficult to detect and analyze *in vivo* and the genes involved in the apoptosis process *in vivo* are also important in cell culture. The use of cell culture systems allows us to detect small fluctuations in the mRNA abundance of specific transcripts, without the masking effects of other cell types. We are also able to tightly control the environmental conditions to which the cells are exposed and eliminate tissue interactions and other variables inherent to *in vivo* studies that may confound the experiment. For each single model system, we use a comparison between cells treated with hormone receptor blocking drug and those that have not been treated to study how the molecular environment changes in response to drug-induced apoptosis sensitivity. In the current study, the selected drug treatments induce an early response of apoptosis sensitivity. By comparing how MCF7 cells respond to TAM and ICI treatment and comparing these to the PC346C response to CAS treatment, we hope to discover genes that are regulated in a concordant fashion between these conditions. We have also included here a comparison with MCF7 cells treated with the apoptosis-inducing factor TNFα that sensitizes the cells to apoptosis through a non-hormone receptor mediated pathway. Those genes that are concordantly regulated by TNFα and the hormone receptor blocking treatments are more likely to represent genes involved in apoptosis sensitivity phenotype rather than occurring as another effect of hormone receptor blockage.

In this study we compare two approaches to identify genes involved in apoptosis. The first approach is a standard n-fold based comparison of MCF7 cells treated with and without TAM. The purpose of this is to demonstrate n-fold analysis and the traditional identification of gene candidates. The second is our proposed comparative functional genomics approach. Here we look for concordant regulation of gene expression based on absolute differences in gene expression levels: any detectable difference in mRNA abundance is considered to be potentially biologically significance. We present data comparing the molecular response to hormone receptor blockage in estrogen-dependent breast cancer cells with hormone receptor blockage in androgen-dependent prostate cancer cells and with TNFα-treated breast cancer cells. All treatments induced apoptosis sensitivity. In our comparisons the central focus is always MCF7 cells treated with the SERM TAM. As diagrammed in Figure 1A, genes that are regulated by TAM, ICI, CAS and TNFα are of a number of distinct classes. We were interested in those genes represented by the black area in the diagram, those genes that have concordant regulation in all four model systems. By comparing all four treatments, we were able to identify those genes that are potentially most important for the induction of apoptosis sensitivity because they are regulated in the same fashion in all the model systems studied.

Materials and Methods

Cell Cultures:

MCF7 human mammary cancer cells were maintained in DMEM/F12 supplemented with 5% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C with 5% CO_2 and 95% air in Nuaire™ IR AUTOFLOW Automatic CO_2 Water-Jacketed Incubator (Nuaire, Inc.). The SERMs TAM and ICI were used at concentration of 5 μM and 10 μM respectively. TNFα was used at a concentration of 1 μM.

PC346C human prostate cancer cells were maintained in DMEM/Ham's F12 (1:1) supplemented with 2% charcoal-stripped calf bovine serum, 1% Insulin-Transferrin-Selenium-G supplement (ITS-G; Life Technologies), 0.1 mg/mL BSA, 20 ng/mL EGF, 1.4 μM hydrocortisone, 1 nM T3, 0.1 mM phosphoethanolamine, 50 ng/mL choleratoxin, 0.1 μg/mL fibronectin, 20 μg/mL fetuin, 10 nM testosterone, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C with 5% CO_2 and 95% air in Nuaire™ IR AUTOFLOW Automatic CO_2 Water-Jacketed Incubator (Nuaire, Inc., Plymouth, MN). The anti-androgen Casodex (Bicalutamide) was used at a concentration of 50 μM.

Cell Number

For determination of cell number, cells were plated in each well of 24-well plate (Corning Inc., Corning, NY) 24 hours prior to treatment. After treatment, cells were washed with phosphate buffered saline (PBS) and fixed in 500 µL 0.2% glutaraldehyde in water for 30 min and stained with 500 µL 0.1% Crystal Violet (Sigma, St Louis, MO) in water for 30 min at room temperature. Plates were destained with water and air dried at room temperature over night. Cells were then incubated with 0.2% Triton X-100 (t-octylphenoxypolyethoxyethanol; Sigma) in water for 1 hour at room temperature and absorbance of the solution from each well was measured at 590 nm using Victor^2 1420 Multilabel Counter (Perkin Elmer Life Sciences, Wellesley, MA).

TUNEL Analysis

For analysis of DNA fragmentation, MCF7 cells treated with 10 µM of TAM or ICI for 48 hours were harvested by trypsinization, fixed with 2% formaldehyde and permeabilized with 70% ethanol. 3'-OH DNA ends were labeled with Terminal Transferase kit (Roche, Mannheim, Germany) and detected with BrdU staining kit (Phoenix Flow System, San Diego, CA). All samples were analyzed using an EPICS XL Flow Cytometer and were modeled with the Multiplus AV software (Phoenix Flow Systems).

Macroarray analysis:

Plasmid stocks of the 96 clones used were taken at random from an archive of clones previously purchased from Research Genetics (Invitrogen, Carlsbad, CA). The clones represent genes involved in general housekeeping, cell cycle, apoptosis, cell stress, signal transduction, DNA repair and some novel genes. Each stock was plated for single colony isolates and plasmid was prepared using a spin mini-prep plasmid kit (Qiagen, Mississauga, Canada). Identities of the clones were confirmed using sequence analysis (DYEnamic ET terminator kit, Amersham Biosciences, Little Chalfont, UK).

PCR products were generated from the plasmid stocks using standard T3, T7 and SP6 primers whose sequences flank the cloning site as appropriate. These PCR products were separated by gel electrophoresis and purified using the QIAquick gel extraction kit (Qiagen, Mississauga, Canada). The purified PCR products were then double spotted onto nylon membrane (BioDyne B, Pall Corp., Port Washington, NY) using a 96 pin arraying device (V&P scientific, San Diego, CA). Sufficient copies of the arrays for the experiment were made at the same time. The DNA on the nylon membrane was denatured in 0.4 M NaOH for 1 min, neutralized in 0.5 M Tris-HCl, pH 7.5 for 1 min. The membranes were allowed to air dry and then the DNA was UV crosslinked to the membrane using a Stratalinker 1800 (Stratagene, La Jolla, CA). Protocols used to perform the array analysis are summarized in Figure 1B.

RNA preparation

Media was removed from the cell cultures grown with or without treatment for 48 hours and replaced with Trizol reagent (Invitrogen, Carlsbad, CA). RNA from samples that had been grown with or without treatment for 48 hours were selected for analysis based on the TAM treated cell concentration results, suggesting that at 48 hours significant apoptosis is not yet occurring. The lysed cells were transferred to polypropylene tubes and frozen pending RNA isolation from all samples as per the standard Trizol protocol. Isolation of high quality RNA was confirmed by formaldehyde-agarose gel electrophoresis (data not shown).

cDNA

First strand cDNA was made from total RNA using PowerScript-RT (BD Biosciences, Palo Alto, CA) and polyT primer. The strand switching property of this RT was used to incorporate a complementary to universal primer sequence at the 3' end of the first strand cDNA. This universal primer was then used to initiate synthesis of the second strand using Taq polymerase. Random prime labeling was used to generate α^{32}P-dCTP labeled cDNA to that was used to probe the arrays. Unamplified cDNA was used to maximize the precision of the experiment at the expense of sensitivity.

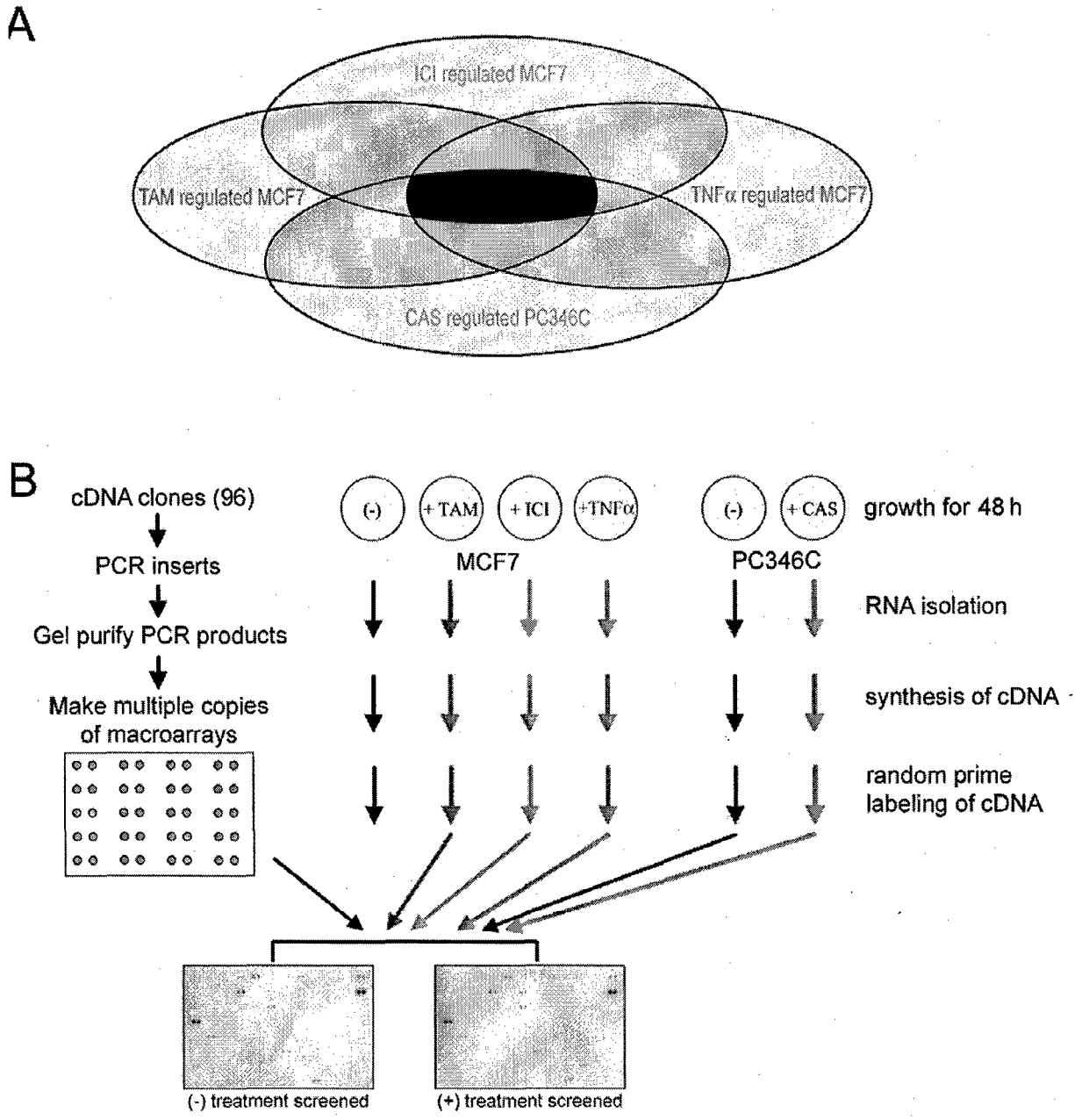

Figure 1. (A) Venn diagram describing the theoretical relationship of molecular changes induced by TAM, ICI, TNFα and CAS. Each oval represents the genes that are under the control of the process labeled within the oval. The area where two ovals overlap represents genes that are controlled by both processes. The genes that we are interested in using comparative functional genomics are those represented by black area in the diagram (B) Illustration of the methods used. Multiple copies of the macroarray were made from purified PCR insert products from plasmid clones. Cells were grown and treated as indicated for 48 hours. RNA was extracted from these cultures, double stranded cDNA was synthesized and radioactively labeled by random prime labeling. Different copies of the array were screened with a different labeled cDNA population from the cultures. Shown in the figure is a representative screening of MCF7 cells treated and untreated with TAM.

Hybridization

Arrays were screened with cDNA probes (MCF7 48 h control, MCF7 48 h TAM treated, MCF7 48 h ICI treated, MCF7 48 h TNFα treated, PC346C 48 h control and PC346C 48 h CAS treated). Prehybridization was performed using 14 ml Hybrisol II (Serologicals Corp., Norcross, GA) at 65°C for 2 hours. Hybridizations were performed in 7 ml Hybrisol II at 65°C for 18 hours. The hybridization solution contained 1.2 million DPM of probe per 7 ml of hybridization solution. Washes were done with the highest stringency being two 15 min washes with 0.1X SSC, 0.1% SDS at 65°C. Blots were stabilized in 2X SSC and wrapped in cellophane and exposed to autoradiography film (Hyperfilm MP, Amersham Biosciences, Little Chalfont, UK) and developed after a suitable period of exposure.

Data analysis

Autoradiographs were scanned using an Epson 1660 Photo Perfection scanner. Images were straightened and cropped using Adobe Photoshop v7 for Windows. Calculation of background intensity, net spot intensity and volume in pixels were done using the histogram function in Adobe Photoshop v7. Analysis of these densitometry results was performed using Microsoft Excel v8 for Windows. A global mean method was used to normalize spot intensities between arrays. In brief, this was accomplished by generating a normalization factor for each array by totaling the net intensity values of all spots within each array and dividing the average for all arrays by each array total. The net intensity values for each spot on the array were then multiplied by the normalization factor for the array to achieve a normalized net intensity.

Results

Treatment with hormone receptor blocking causes apoptosis sensitivity

MCF7 cells treated with 5 μM TAM for 96 hours showed a dramatic reduction in cell number as compared to the control (Figure 2A). The number of cells after TAM treatment for 96 hours was approximately 30% of the untreated control. The number of cells that persist after a 48 hour treatment was 73.2% of the untreated control. This parallels data produced by TUNEL labeling and flow cytometry that showed 19.93% of MCF7 cells treated with TAM for 48 hours had fragmented DNA (Figure 3).

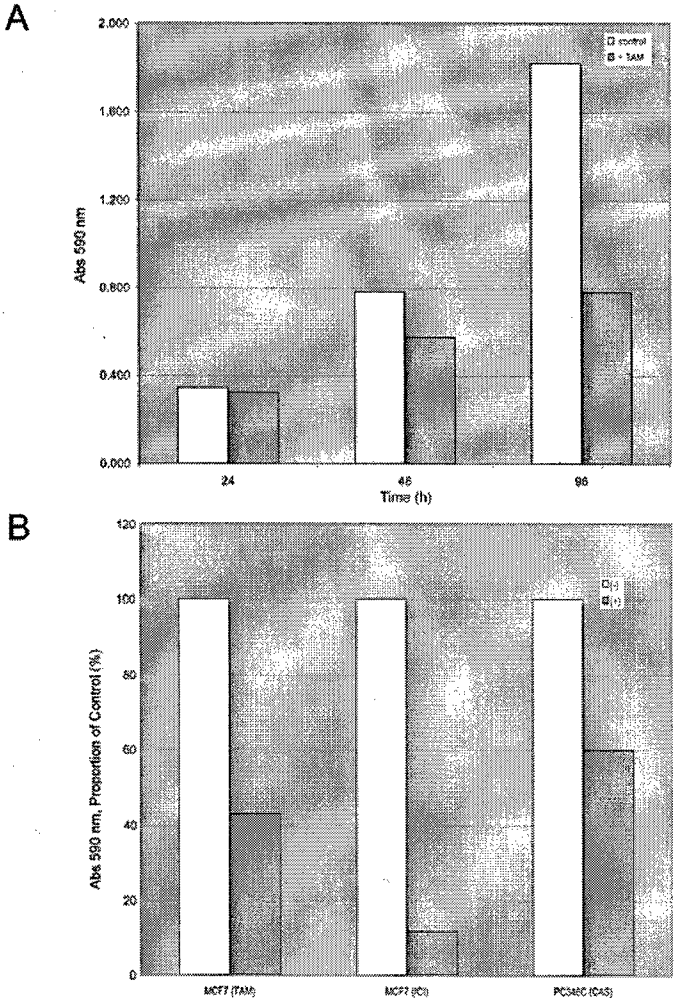

Figure 2. Treatment of cells with TAM, ICI, TNFα and Casodex lead to a decrease in cell numbers. (A) Cell densities of control MCF7 cells (untreated) or MCF cells treated with TAM (5 μM), for 24, 48, 72, 96 hours. There is a significant effect on cell numbers as early as after a 48 h treatment. (B) Normalized comparison of cell densities after 96 hour treatments with and without hormone receptor blockers. MCF7 cells and PC346C (prostate cancer) cells were treated with and without hormone receptor blockers (MCF7 cells with 5 μM TAM and 10 μM ICI, PC346C cells with 50 μM Casodex). White bars are indicative of control cell densities which were set at 100% (untreated). Gray bars are indicative of cell densities normalized to control levels after treatment with hormone receptor blockers for 96 hours.

MCF7 cells treated with ICI gave similar results to those seen with TAM treatment (Figure 2B). These observations were consistent with the fact that the MCF7 cells are dependent upon estrogen for normal function and that both

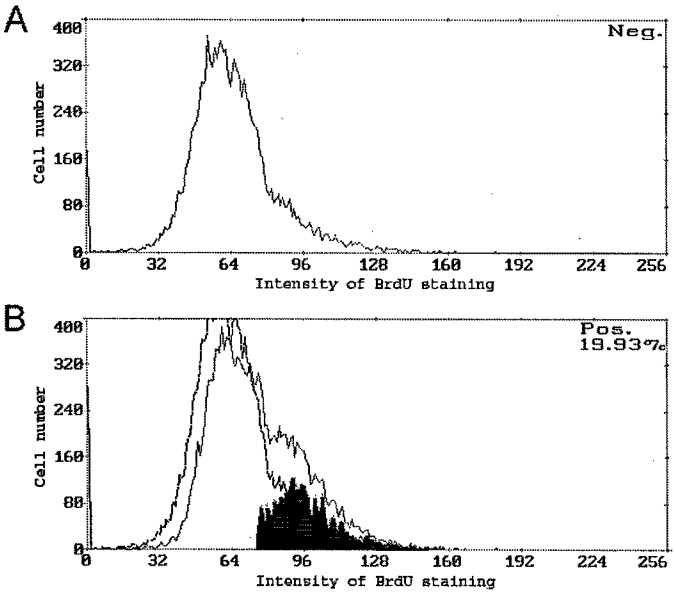

Figure 3. Reduction in the number of MCF7 cells within 48 hours of treatment with TAM is due to apoptosis as indicated by cells with increased amounts of BrdU label with TUNEL labeling. (A) cell sorting results for untreated cells that have been TUNEL labeled. (B) tunnel labeled cells that have been treated with TAM and sorted using FACS analysis. 19.93 % of the cells showed TUNEL labeling over the control.

TAM and ICI act as antagonists of estrogen receptors. TNFα treatment of MCF7 cells also resulted in a dramatic reduction in cell number compared to control at 96 hours (data not shown). When treated with the anti-androgen CAS, PC346C cells showed a decrease in cell number as compared to untreated controls (Figure 2B). In addition, it has been shown that MCF7 cells treated with TAM, ICI or TNFα and prostate cancer cells treated with CAS become apoptotic.[13-16] We therefore concluded that the majority of the treated cells that contributed to the RNA sample had an apoptotic sensitivity phenotype.

n-fold analysis of gene expression profiles

Comparisons of mRNA expression profiles of TAM treated and control treated MCF7 cells for 48 hours were performed indirectly using custom-made macroarrays. A 48 hour treatment was chosen because it represents an early response state prior to the induction of apoptosis in the majority of the cells. The screen with both probes used in the differential screen identified 16% (15/96) of the clones showing quantifiable levels of signal (Table 1).

An arbitrary n-fold cutoff point of 1.5 fold, a state in which the hybridization levels in the treated sample is at least 50% higher or lower than the levels observed for the untreated sample, was used as the criteria to select out putative differentially expressed genes (Table 1). When TAM treated MCF7 cells were compared to control, the genes identified as differentially expressed would be ARHA, CACNA1F and PCNA. If the focus of our study had been to use n-fold analysis to identify differentially expressed genes, these would then be further studied and very well may have an involvement in the apoptosis phenotype.

As part of our study, copies of the same macroarrays were used to analyze expression changes with different treatments of the MCF7 cells and PC346C cells (Figure 4). When each individual treatment was analyzed using n-fold analysis at a n-fold cut off of 1.5 fold, the differentially expressed genes were as follows: (1) ICI-treated MCF7 differential genes (HSPD1, EIF3S6, PCNA). (2) CAS-treated PC346C differential genes (Hs.28426, ARHA, HSPD1, EIF3S6, TRA1). (3) TNFα-treated MCF7 differential genes (Hs.28426, HSPD1, EIF3S6, TRA1). The expression levels in control and treated cell samples are shown in Figure 4. With the criteria of a 1.5 fold change, there were no genes that are consistently differentially regulated in all 4 conditions.

Functional genomics comparison of gene expression profiles

In our comparative functional genomics analysis, any changes in the expression levels of the genes on the array were considered potentially biologically significant. In the set of 15 genes that were relevant to our analysis, all showed a high level of expression in untreated cells. Thus a small difference in the expression level of these genes after treatment may not have registered as a significant change based on fold differences but could very well have represented a significant difference when considering the number of active transcripts that were present in the cells or in particular cells. The mechanisms that activate and inhibit apoptosis are carefully balanced. Small perturbations in the concentrations of molecules affecting this balance can have a cascading effect. Therefore any change in a molecule involved in apoptosis sensitivity can be biologically significant.

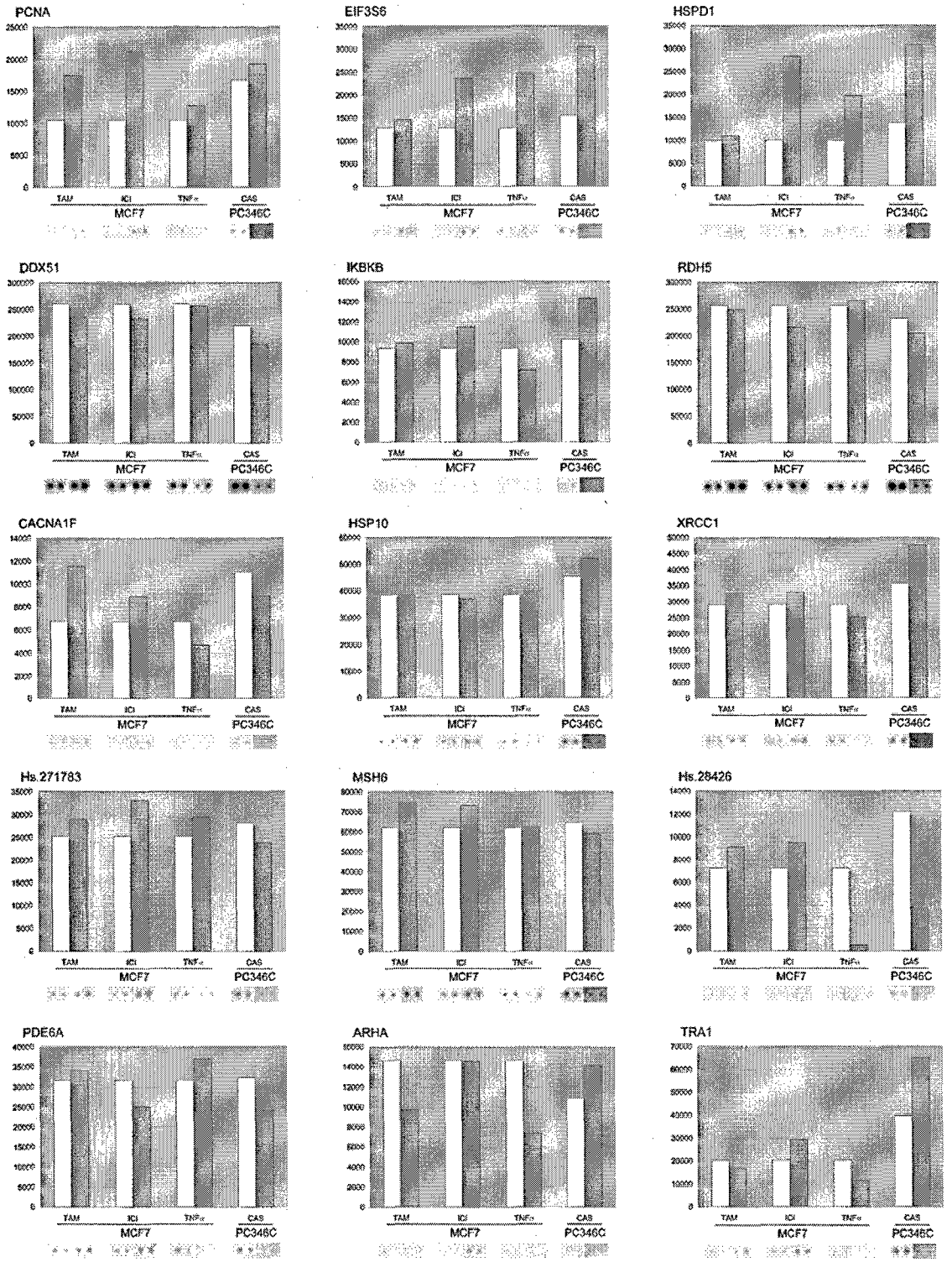

Figure 4. Expression profiles with TAM and ICI treatment of MCF7 cells and CAS treatment of PC346C cells. Shown are the expression profiles for the 15 clones that were detected with our screens. Gray bars represent the normalized hybridization intensities detected by probes derived from cell cultures treated with the chemical in question for 48 hours. White bars represent the normalized hybridization intensities by the probe derived from the corresponding untreated controls (48 hours). Below each chart the cell type and treatments are indicated as well as a figure of the hybridization spots from the autoradiographs of the screened arrays. Genes that show similar expression profiles are grouped together as indicated by a letter designation and outlined by a solid line.

Table 1. Normalized intensities of measurable spots from the macroarrays screened for MCF7 TAM induced differential gene expression. Names of the genes represented by the PCR product spotted on the array are indicated on the left column of the table. In the right column are the n-fold values calculated for treated/control. Those n-fold values equal to or greater than 1.50 or 1/1.50 are bolded.

Gene	MCF7 (-)	MCF7 +TAM	n-fold
RDH5	256876	248943	0.97
CACNA1F	6720	11530	**1.72**
EIF3S6	12700	14572	1.15
PCNA	10425	17420	**1.67**
MSH6	61913	74325	1.20
XRCC1	29104	32628	1.12
HSPD1	9896	10950	1.11
HSP10	38524	38462	1.00
TRA1	20373	16756	0.82
IKBKB	9313	9871	1.06
PDE6A	31539	33927	1.08
ARHA	14605	9705	**0.66**
Hs.271783	25189	28978	1.15
DDX51	259902	235394	0.91
Hs.28426	7250	9125	1.26

In Figure 5. the comparison of treated to control expression is categorized as up-regulated (green), down-regulated (red) or no difference (yellow). In the analysis of expression trends, there was a consistent increase in HSPD1, EIF3S6, and PCNA expression levels after drug treatment in all cases. There was also a consistent decrease in the expression of DDX51. This group of gene responses was considered as potentially involved in the apoptosis sensitivity phenotype that was common between all the treatment groups. IKBKB, XRCC1 and CACNA1F were consistently up-regulated and RDH5 down-regulated after TAM and ICI treatment of MCF7 cells and CAS treatment of PC346C cells. The regulation of these genes was therefore considered to be a common hormone receptor mediated response. Expression of genes such as Hs.271783, MSH6, Hs.28426 were induced after TAM and ICI treatment of MCF7 cells. The induction of these genes was considered to be an estrogen receptor mediated response.

Discussion

In the first part of our analysis, a n-fold analysis at a 1.5 fold differential abundance cut-off value was used to show that PCNA, CACNA1F, and ARHA were differentially expressed as defined by a differential screen with TAM treated and untreated MCF7 derived cDNA probes. This was a simple pairwise analysis of how TAM changes the expression profile of MCF7 cells in culture. Traditionally, these three genes would have been further examined to identify what their function might be in an apoptosis susceptible phenotype. With the comparative functional genomics approach, changes in gene expression at any level may have some biological significance if they are conserved in different model systems induced to undergo the same biological process. We were therefore looking for consistent up-regulation or consistent down-regulation of genes after exposure to different stimuli (different drug treatments) that produced similar phenotypes (cells with apoptosis sensitivity) in different inducible apoptotic models. By looking at a second SERM, the pure anti-estrogen ICI, that also blocked the function of the estrogen receptor, the criteria was developed to be able to define which genes might underlie an estrogen receptor mediated response including sensitivity to death signals. When the response of PC346C cells to CAS to our list of model systems we expanded our study to address which genes are common in more generalized hormone receptor blockage responses. Finally with examination of the effects of TNFα treatment that induced apoptosis via cell surface receptors and a series of signal transduction cascades, those genes were identified that mediated common responses between these treatments and therefore more likely to be involved in the common apoptosis sensitivity phenotype. The multi-model comparative approach thus allowed us to develop certain insights that a simple two-way differential analysis did not. Given the diversity of the model systems, we could begin to unravel those molecular events that underlie a receptor mediated apoptosis sensitivity phenotype.

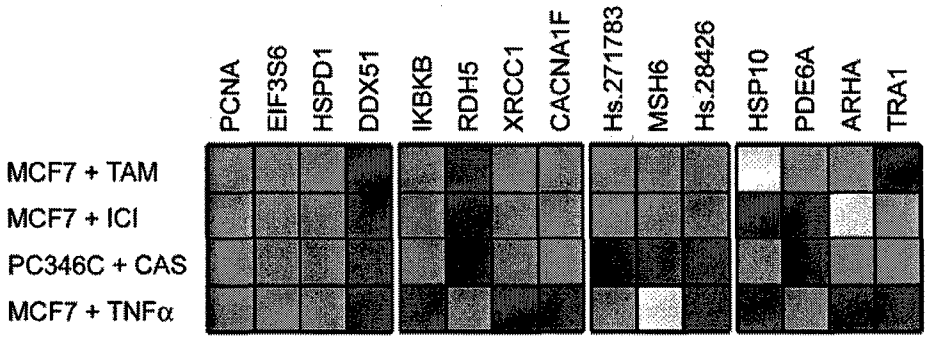

Figure 5. Analysis of absolute changes in gene expression seen with the 4 treatments that induce apoptosis sensitivity. Green indicates an up-regulation with treatment, red indicates a down-regulation and yellow indicates no change in expression. In total 4 genes were found to have a consistent change in all 4 ACD inducible model systems (PCNA, EIF3S6, HSPD1 and DDX51). Three of these genes (EIF3S6, HSPD1 and DDX51) were not detected using a n-fold analysis.

Our focus for all the comparisons was MCF7 cells treated with TAM. With the n-fold analysis PCNA, ARHA and CACNA1F were identified as differentially regulated by TAM treatment. The role of PCNA in apoptosis is discussed below. ARHA is primarily involved in actin cytoskeletal rearrangements and metastatic invasion in response to integrin activation.[17-19] There are conflicting reports as to the role of ARHA in inducing apoptosis therefore it is questionable whether ARHA activation is directly related to apoptosis.[20,21] The role of ARHA as an oncogene may be more related to invasion and metastasis rather than apoptosis regulation. CACNA1F is a type II voltage dependent calcium ion channel and is expressed *in vivo* almost exclusively in eye tissues.[22,23] It is therefore unlikely to be a conserved and important member in the process of apoptosis. These results demonstrated that n-fold analysis does not reliably detect genes that are involved in a particular process such as apoptosis.

Comparison of the n-fold analysis differentially expressed genes with TAM treatment with the results of our comparative functional genomics analysis shows only PCNA on both lists. In addition, HSPD1, EIF3S6 and DDX51 show similar changes in expression patterns in all the model systems examined but were not identified in a differential screen for responses to TAM treatment using a 1.5 n-fold significance criterion.

The known functions of PCNA, EIF3S6, HSPD1 and DDX51 are consistent with a role in an ACD phenotype. PCNA is a protein that is regulated by cell cycle and is related to DNA repair.[24,25] It is involved in DNA replication during S-phase and is ubiquitinated during DNA repair. The role of PCNA in cancer-associated phenotypes has not been completely described, but may be related to its role in DNA repair and regulation of the cell cycle. It has also been shown that cells that are actively proliferating can be induced to undergo apoptosis by blockage of the cell cycle, therefore PCNA upregulation in response to hormone receptor blocking treatment may help to sensitize these cells to apoptosis.[26] Eif3S6 is a protein involved in the initiation of translation and as such could be involved in modulating proteome profiles within the cell.[27] Changes in the levels of Eif3S6 protein could change which mRNA transcripts are selected for translation. EIF3S6 has been associated with breast cancer because the EIF3S6 locus is a common site for the integration of the MMTV virus resulting in mammary tumors in mice.[27,28] HSPD1 is a heat shock protein that acts as a mitochondrial molecular chaperone and is the homologue of archetypal bacterial chaperone GroEL from *Escherichia coli*.[29] The consistent upregulation of HSPD1 with the treatments as shown in Figure 5 suggests that these treatments may be inducing cellular stress within the mitochondria of these cells. Mitochondrial instability has been associated with TAM treatment and with the apoptosis induction.[7] DDX51 contains a DEAD box (Asp-Glu-Ala-Asp) sequence motif[30] that has been associated with genes involved in DNA binding, DNA replication and transcription initiation.[31] If Ddx51 also has an effect on these cellular processes then changes in the level of its expression could mediate changes in the molecular environment leading to increased sensitivity to induction of apoptosis.

Conclusions

This study has described two key observations. The first is PCNA, EIF3S6, HSPD1 and DDX51 could have important roles in the apoptosis sensitivity associated with receptor mediated responses in breast cancer cells and prostate cancer cells. This role is supported by what is already known about these genes and their roles in apoptotic phenotypes. Second, and more importantly, we present a novel way to examine and dissect gene expression and

provide a novel comparative functional genomics strategy to assay for genes that may be relevant to a specific biological process. The genes identified by our comparative approach may not have been identified through more traditional differential approaches to gene selection. Although the roles of the genes identified in our study need further functional characterization and it still is unclear as to whether small differences in mRNA production lead to effects on proteins, enzymes or metabolites, we feel that the comparative functional genomics approach is useful for identifying genes potentially involved in apoptosis and has broader applicability to other biological processes.

Acknowledgements

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Commentary

On the Trail of Cell Death Pathways in Prostate Cancer

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KEY WORDS

prostate cancer, PI-3 kinase, PDK1, TPCK

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Commentary to:

Multiple Effects of N-α-Tosyl-L-Phenylanlyl Chloromethyl Ketone (TPCK) on Apoptotic Pathways in Human Prostatic Carcinoma Cell Lines

Oskar Rokhlin, Natalya Guseva, Agshin Taghiyev, Rebecca Glover and Michael Cohen

Most prostate tumors are slow growing and have a small fraction of the cells in S phase, rendering primary and most metastatic tumors resistant to standard chemotherapy regimens. In consequence the main focus in the development of effective treatments has been on therapies that interfere with the cell survival pathways and induce apoptosis in the tumor cells. Like most cells, prostate cancer cells have both intrinsic and extrinsic pathways that regulate cell survival and cell death (summarized in Fig. 1). While it is well established that androgen ablation or anti-androgen treatment induce tumor regression, most probably through the activation of the mitochondrial (intrinsic) pathway, the pathway linking interruption of signaling through the nuclear receptor and mitochondrial permeability transition (PM) has yet to be fully elucidated.[1] Furthermore the appearance of more aggressive, hormone refractory tumors after anti-androgen therapy indicates that interruption of the androgen signaling pathways may be incomplete and have undesired long term effects,[2] highlighting the need for better adjuvant therapies or new therapies based on other cell death pathways.

Other potential targets for treatment of prostate cancer do exist. Prostate cancer cells express a family of membrane associated death receptors and respond to external signaling molecules include TNFα (tumor necrosis factor α), TRAIL (TNF-related apoptosis-inducing ligand) and Fas L (Fas ligand). These receptors, and the downstream signaling pathways emanating from them, have emerged over the last few years as promising targets for therapeutic intervention.[3-5] The authors of the accompanying report, and others, have shown that prostate cancer cells lines display a wide range of sensitivities to the major ligands which interact with the cognate cell surface receptors, including TNFR1, TRAIL-R1 and TRAIL-R2 and Fas.[6-10] The differences in sensitivity to the ligands has variously been attributed to differences in Bcl-2 levels,[11] differences in IκB and NFκB activation,[12-16] PTEN expression,[17] constitutive activation of the AKT pathway[18] and survivin levels induced through β1-integrin signaling.[19] The authors themselves have previously shown that formation of the TRAIL-DISC in LNCaP cells is androgen dependent.[20] While there are probably a number of biological mechanisms underlying the selective resistance of cancer cells to the death inducing ligands, the TRAIL-mediated pathway leading to apoptosis has emerged from these studies as a very promising target for intervention. Understanding the underlying regulation of the signaling pathways downstream of the death receptors and the interactions with the survival pathways will be critical for the development of effective therapies. The accompanying paper describes these interactions a particular focus on the TRAIL-mediated apoptotic pathway and the PDK1/AKT pathway in androgen responsive LNCaP and androgen insensitive PC-3 cells. Using a variety of inhibitors of the TRAIL-mediated and receptor tyrosine kinase-mediated pathways: wortmannin, a specific inhibitor of the AKT pathway; MG132, a serine protease and proteasome inhibitor; and TPCK, an inhibitor of PDK1/AKT pathway, the current manuscript carefully dissects the regulation of TRAIL induced cell death in prostate cancer cells, and the interactions between the cell death and cell survival pathways in these cells.

Activation of TRAIL-R1 or TRAIL-R2 leads to the formation of the "Death-Inducing Signaling Complex" (DISC) on the cytoplasmic domain of the receptors. The DISC utilizes FADD predominantly as the adapter to recruit pro-caspase 8. Autocatalytic activation of pro-caspase 8 can lead directly to the proteolytic activation of pro-caspase 3, and the rapid initiation of DNA fragmentation (the so called extrinsic pathway), or it can lead to the initiation of mitochondrial membrane transition through the insertion of t-Bax into the mitochondrial membrane (the intrinsic pathway), formation of the apoptosome and slower activation of pro-caspase 3 and DNA fragmentation. Although it is not yet established what leads to the choice of which pathway is selected after assembly of the DISC, it has been suggested that it may be related to the amount of pro-caspase 8 recruited to the DISC which in turn may be dictated by other components required for the assembly of the DISC such as acid sphingomyelinase,[21] selective recruitment of the inhibitor cFLIP to

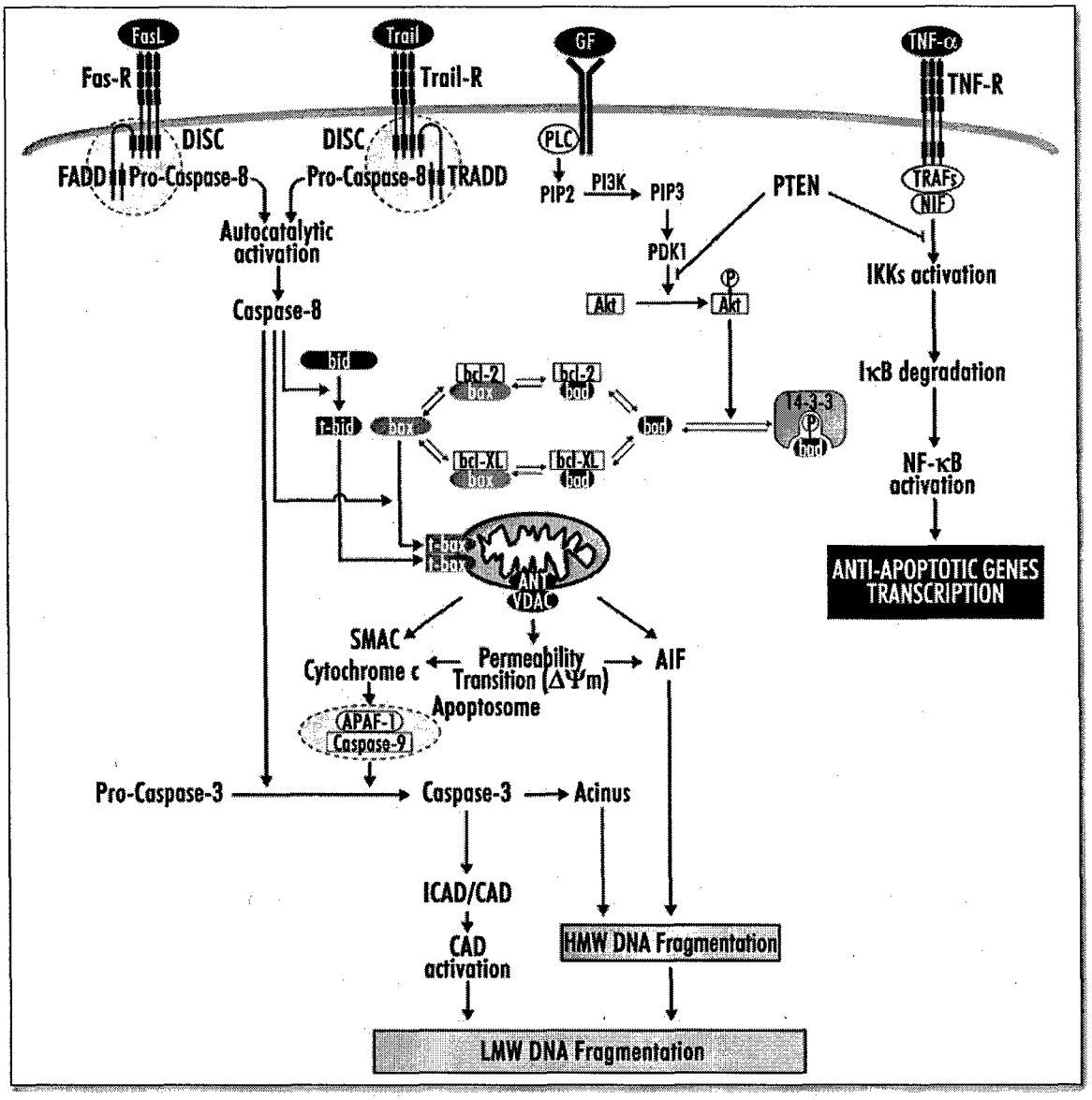

Figure1: Interaction between death receptor-mediated cell signaling and receptor tyrosine kinase mediated survival pathways

the DISC,[22,23] or more fundamental changes in the biosynthesis and surface expression of specific death receptors.[24]

Activation of caspase 8 is necessary but not sufficient for the induction of cell death, inhibition of the receptor tyrosine kinase mediated cell survival pathways is also necessary to ensure that cells complete the apoptotic process. Under normal conditions signaling through receptor tyrosine kinases ensures that Bax is maintained as an inactive heterodimer in association with Bcl-2, Bcl_{X-L} or another member of the anti-apoptotic Bcl-2 family. Signaling through receptor tyrosine kinases, or through integrins[25] induces the release of PtdIns(4,5)P2 (PIP2), which is converted by phosphatidylinositol 3-kinase (PI3K) to form the second messenger PtdIns(3,4,5)P3 (PIP3). This phosphorylation is necessary for the binding of these membrane lipids to the pleckstrin-homology domain of AKT, and its recruitment to the membrane where it is activated by 3-phosphoinositide-dependent kinase (PDK1).[26] Activated AKT phosphylates Bad, which is sequestered by 14-3-3, ensuring cell survival. Inhibiting this signaling pathway at any point results the dephosphorylation of Bad, and the disruption of the equilibrium between the pro- and anti-apoptotic heterodimeric partners, exposes monomeric Bax to caspase 8 (produced as a consequence of TRAIL-R activation and assembly of the DISC). This leads to the formation of t-Bax which integrates into the outer mitochondrial membrane, releasing cytochrome c and SMAC/Diablo into the cytoplasm, triggering the formation of the apoptosome and subsequent proteolytic activation of pro-caspase 9 and pro-caspase 3 and ultimately both high molecular (HMW) weight and nucleosomal (LMW) DNA fragmentation.

The accompanying paper shows that TPCK clearly has important effects on both the cell death and cell survival pathways. Comparison the effects of TPCK on the components of the cell death pathways in LNCaP cells has shown that TPCK preferentially downregulates and inhibits TRAIL-mediated caspase activation, most likely by inhibiting TRAIL-DISC formation. Since TPCK does not affect the formation of Fas-DISC, this suggests that the composition of the

two DISC complexes is somehow different, and that the target of TPCK is not one of the components in common between the two complexes. It may be possible to exploit this ability of TPCK to discriminate between DISC complexes to develop receptor selective therapies. Despite these effects on the initiating complex TPCK has minimal effects on the level of caspases 3, 7, 8 or 9 in PC-3 cells, however as the authors show these caspases can be activated by other apoptotic inducers, making TPCK an attractive candidate for adjuvant approaches. The effects of TPCK on the cell death pathway alone might be expected to decrease efficacy of TRAIL-mediated cell death, however the effects of TPCK on the cell survival pathways actually render the cells more sensitive to TRAIL. TPCK blocks the phosphorylation and activation of PDK1 in LNCaP which attenuates the phosphorylation of AKT. In the absence of activated AKT, the level non-phosphorylated Bad increases, disrupting the homostasis of the Bcl-2 heterodimers, and promoting the release of SMAC and cytochrome c from the mtiochondria. The mechanism of this effect on the AKT pathway has not been elucidated. While it is clear that one of the earliest effects of TPCK is the decreased phosphorylation of PDK1, and the simple explanation for the effects of TPCK may rest on its ability to inhibit the autophosphorylation of PDK1, it is also possible that TPCK alters the metabolism of PIP3(which is necessary for both the recruitment of AKT to the membrane and activation of PDK1), by activating PTEN or SHIP2 which metabolizes PIP3 to PtdIns(3,4)P2.[27]

The authors also show that TPCK effectively downregulates the expression of the unoccupied androgen receptor in a manner that is only minimally blocked by a pan-caspase inhibitor. Since TPCK does not appear to be as effective an inhibitor of the proteasome as MG-132, it probable that the drug is enhancing proteasome degradation of the AR, although there is no indication from the current manuscript as to the mechanism by which this might occur. Since both AR and p53 (which is also downregulated in LNCaP cells by TPCK) are regulated by ubiquitination it is possible that TPCK also has unexplored effects on the ubiquitination pathways responsible for the regulation of a variety of transcription factors.

In summary this paper highlights encouraging data regarding the potential to develop therapies for both androgen dependent and independent prostate cancer that are based on manipulation of the cell death membrane receptors, that can be used independently of anti-androgen therapy or as an adjuvant therapy. This promises to be a fruitful line of investigation.

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